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Citation for published version:

Lachapelle, J & Colegrave, N 2017, 'The effect of sex on the repeatability of evolution in different environments', *Evolution: International Journal of Organic Evolution*, vol. 71, no. 4, pp. 1075-1087.
<https://doi.org/10.1111/evo.13198>

Digital Object Identifier (DOI):

[10.1111/evo.13198](https://doi.org/10.1111/evo.13198)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Evolution: International Journal of Organic Evolution

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The effect of sex on the repeatability of evolution in different environments

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Running head: Sex and the repeatability of evolution

Keywords: Recombination, selection, historical contingency, chance, experimental
evolution, *Chlamydomonas reinhardtii*

Data archival: The data associated with this manuscript will be archived in DRYAD
when accepted for publication.

Abstract

The adaptive function of sex has been extensively studied, while less consideration has been given to the potential downstream consequences of sex on evolution. Here we investigate one such potential consequence, the effect of sex on the repeatability of evolution. By affecting the repeatability of evolution, sex could have important implications for biodiversity, and for our ability to make predictions about the outcome of environmental change. We allowed asexual and sexual populations of *Chlamydomonas reinhardtii* to evolve in novel environments and monitored both their change in fitness and variance in fitness after evolution. Sex affected the repeatability of evolution by changing the importance of the effect of selection, chance and ancestral constraints on the outcome of the evolutionary process. In particular, the effects of sex were highly dependent on the initial genetic composition of the population and on the environment. Given the lack of a consistent effect of sex on repeatability across the environments used here, further studies to dissect in more detail the underlying reasons for these differences as well as studies in additional environments are required if we are to have a general understanding of the effects of sex on the repeatability of evolution.

Introduction

The ubiquity of sexual lineages among eukaryotes is a long-standing problem in biology (Smith 1978; Bell 1982). Extensive research has examined the adaptive function of sex, that is the mechanisms for its origin and maintenance over evolutionary time (Lively and Morran 2014; Becks and Alavi 2015). However, less consideration has been given to the potential downstream consequences of sex on evolution, such as changes in genome modularity and architecture, population differentiation, or evolvability. While these consequences may or may not have any adaptive significance, they can potentially have important implications for evolution. Here we investigate one potential downstream consequence of sex: the effect of sex on the repeatability of evolution. By altering the repeatability of evolution, sex could affect the predictability of evolution, and have long-term consequences for rates of diversification.

The repeatability of evolution depends on the relative importance of natural selection as the deterministic driver (Fisher 1930; Muller 1932; Gerrish and Lenski 1998; Desai and Fisher 2007), chance as the stochastic driver (Lenski and Travisano 1994; Wiser et al. 2013), and ancestry as the source for unpredictable constraints (Weinreich et al. 2005) on further evolutionary change. The relative roles of these components in adaptation can be examined experimentally by allowing initially genetically identical replicate populations to evolve in identical environments. If replicates reach similar evolutionary end points then evolution has been highly repeatable, whilst differences in end points indicate the role of chance. If the experiment is repeated with different ancestors, the role of ancestral constraints can also be evaluated.

In general, experiments in asexual and initially isogenic populations of microbes show that fitness changes during adaptation to a novel environment are often repeatable (Travisano et al. 1995; Collins et al. 2006; Flores-Moya et al. 2008; Bell 2012b; Spor et al. 2013). However, differences in ancestry, combined with chance events during adaptation, can have a significant effect on the fitness trajectories of evolving populations in some environments (Melnyk and Kassen 2011) and in small populations (Lachapelle et al. 2015). Chance effects also seem to be more important for the evolution of phenotypic traits than for the evolution of fitness itself (Travisano et al. 1995; Collins et al. 2006; Flores-Moya et al. 2008; Bell 2012b).

In sexual populations, the relative contribution of selection, chance, and ancestry is highly variable from study to study (Teotonio and Rose 2000; Teotonio et al. 2002; Kawecki and Mery 2003; Joshi et al. 2003a; Griffiths et al. 2005; Simões et al. 2008; Teotónio et al. 2009; Fragata et al. 2014). However a direct experimental comparison of the repeatability of evolution of sexual and asexual populations has not, to the best of our knowledge, been done.

There are reasons to expect the repeatability of evolution to be higher in sexual populations since recombination allows selection to act independently at different loci (McDonald et al. 2016). That is, when a beneficial mutation appears in an asexual population, its ultimate fate will be strongly influenced by the genetic background that it arises in. A beneficial mutation that arises in a relatively low fitness background will ultimately be lost, whilst the same mutation arising in a better background may fix

(Weismann 1889; Fisher 1930; Muller 1932). As a result, initial chance associations will be relatively important. In contrast, in a sexual population, the beneficial mutation can be selected independently of the background it appears in (Hill and Robertson 1966; Felsenstein 1974; Peck 1994), making such chance associations possibly less important and evolution more deterministic.

We also expect divergence among individuals in sexual populations to be lower than within asexual populations after evolution. In sexual populations, the variance that is generated by one episode of sex (Colegrave et al. 2002) will increase the efficiency of selection, and recombination will separate beneficial mutations from their background, reducing the spread of groups of linked mutations (McDonald et al. 2016). Whereas in asexual populations, competition between beneficial mutations in different individuals, i.e. clonal interference, will slow the fixation of mutations, and hitchhiking of mutations on the background of the beneficial mutation will lead to a greater number of mutations fixed overall (Kao and Sherlock 2008; Lang et al. 2013; McDonald et al. 2016). Hence we expect there will be fewer mutations segregating at any one point and fewer mutations fixed in sexual populations than in asexual populations.

To test these hypotheses, we compare the repeatability of evolution between asexual and sexual experimental populations of the green alga *Chlamydomonas reinhardtii* in four different environments. In particular, we focus on how (1) the efficiency of selection; and (2) chance and ancestral constraints differ between asexual and sexual populations. We estimate the efficiency of selection by measuring rates of fitness change; and we estimate

the role of chance and ancestral constraints by measuring variance in fitness among independent populations. We find that the effects of sex are highly dependent on the environment, with sex enhancing convergence in some environments and divergence in others.

Material and Methods

Base populations

We generated three genetically different starting points by crossing three different pairs of wild-type strains of the haploid green alga *Chlamydomonas reinhardtii*. Ancestry A was generated by using the F1 progeny from a cross between CC-1690 and CC-1691; ancestry B using the F1 progeny from a cross between CC-2342 and CC-2344; and ancestry C using the F1 progeny from a cross between CC-2931 and CC-2937 (Figure 1). These strains have been shown to be genetically (Smith and Lee 2008; Flowers et al. 2015) and phenotypically (Malcom et al. 2015) different, with the average divergence between any pair of strains being about 3% (Smith and Lee 2008; Flowers et al. 2015). Based on population structure analyses, the strains from ancestry A belong to the Laboratory group of strains; strains from ancestry B belong to the West group of strains; and strains from Ancestry C belong to either the Southeast and West groups (CC2391), or the Northeast group (CC2937) (Flowers et al. 2015). The progeny from each cross should retain the genetic signature of their two parents and therefore maintain on average the genetic dissimilarity that was present among parents from each ancestry.

Twelve spores from each ancestry were isolated at random from each pool of progeny, for a total of 36. From now on these spores are referred to as the ancestors. Each experimental line was assembled using eight spores from a given ancestry: the asexual lines contained eight spores of a single mating type (we used spores of mating type - for Ancestry A and C, and spores of mating type + for Ancestry B), whereas the sexual lines contained four spores of mating type + and four spores of mating type -. The asexual and sexual lines from a given ancestry thus shared four ancestral spores. We started our experiment with genetically diverse populations instead of genetically uniform populations because the sexual populations required at least two genotypes (i.e. one genotype of each mating type), and because the larger the number of genotypes sampled from the F1 progeny, the higher the probability that asexual and sexual populations will share the same amount of genetic variance. Indeed, our growth assays show that the ancestral spores used to assemble the asexual lines do not differ statistically from the ones used to assemble the sexual lines in their growth rates across the four selection environments described below (linear mixed model with Satterthwaite approximations to degrees of freedom: $t_{10} = -0.88$, $P = 0.40$). Hence, the mode of reproduction treatment is not confounded with differences in starting points.

Selection experiment

Each ancestral spore was grown individually from a single colony. Once fully grown, the ancestral spores were pooled together to construct each experimental line, and 24 samples (six replicates in each of four environments) of each mixture were used to initiate each replicate line, which were then propagated independently.

For each combination of ancestry and mode of reproduction, we had 6 replicate lines, for a total of $3 \times 2 \times 6 = 36$ independent lines. Each line was propagated in each of four different environments: Bold's minimal medium (referred to as Bold's; Harris 2009); Bold's minimal medium supplemented with $0.435 \mu\text{M}$ Atrazine and $0.250 \mu\text{M}$ S-metolachlor (referred to as Herbicides); Bold's minimal medium supplemented with $7 \text{ gL}^{-1} \text{ Na}_2\text{SO}_4$ (referred to as Na_2SO_4); and Bold's minimal medium supplemented with $5 \text{ gL}^{-1} \text{ NaCl}$ (referred to as NaCl). The environments were chosen to represent a random sample of all possible environments, and because they are easily tractable in the laboratory. Bold's is a standard laboratory medium representing fairly benign conditions. NaCl (Lachapelle and Bell 2012; Lachapelle et al. 2015) and Herbicides (Lagator et al. 2014) have been used in the past to study adaptation in *C. reinhardtii*, and target different aspects of growth (i.e. NaCl generates osmotic and oxidative stresses, while Atrazine targets photosynthesis and S-metolachlor targets the synthesis of long chains of fatty acids). We chose to use 5 gL^{-1} of salt because it has been shown to elicit evolutionary responses within 200 generations (Lachapelle and Bell 2012). We used two herbicides instead of only one to generate a more complex target to selection. We chose to use $0.435 \mu\text{M}$ of Atrazine and $0.250 \mu\text{M}$ of S-metolachlor after preliminary assays with a range of concentrations showed that this combination reduced fitness significantly, but not to an extent where cell densities become lower than the detection limit of the spectrophotometer. Finally, Na_2SO_4 was chosen randomly as its effects on *C. reinhardtii* are unknown. It occurs naturally in lakebeds, in the form of mirabilite in wet or damp environments and in the form of thenardite in arid environments. Similarly to the herbicides, we chose to use 7 gL^{-1} after preliminary assays with a range of concentrations.

Preliminary assays showed that the stressors and the concentrations chosen reduce growth rates to different extents compared to the benign environment of Bold's.

The experiment consisted of vegetative growth cycles interspersed with sexual cycles. The lines were cultured in 24-well plates, with breathable sealing films to ensure even evaporation and air exchange across the plate (except during mating where the plastic lids were used to ensure optimal light intensity), shaken at 180 r.p.m. with a 3 mm rotation diameter. The cultures were maintained in a growth chamber at 24 degrees Celsius, 60% humidity, and 8000 Lux constant lighting.

The sexual cycles were imposed after about 10, 50, 100, 150, 200, and 260 generations of vegetative growth. The protocol for the sexual cycle was imposed on all lines, even on the asexual lines, which were not expected to mate given that they were composed of spores of only one mating type. Cultures were visually inspected under a microscope during each sexual cycle to confirm the absence of mating reactions in the asexual lines. Briefly, at the end of a vegetative growth cycle, the spent media was replaced with nitrogen-free media by centrifuging the cultures. The cultures were left static in nitrogen-free liquid media for approximately 24 hours to allow gametogenesis and mating to occur. After this period, the zygotes and 50 μ L of culture were transferred to an agar plate, or in the case of the asexual lines 50 μ L of culture was transferred to an agar plate. The agar plates were wrapped in aluminium foil and left in the dark for zygote maturation to occur. After four days, mature zygotes were exposed to chloroform vapour for 45 seconds to kill unmated cells, and then placed under the lights for germination. The

asexual lines were not exposed to chloroform but put directly under the lights. After two days in the light, the cells were re-suspended in liquid media and transferred back into the vegetative growth cycles. The cultures were then serially transferred every 3-4 days using a 5% inoculum (100 μ L into 1900 μ L of fresh media). A total of 6 sexual cycles and 60 vegetative cycles were imposed for a total of about 300 generations.

Seven sexual lines (three from the Na₂SO₄ environment and four from the Herbicides environment) went extinct during the experiment because they failed to mate during the sexual cycle. In particular, in the Na₂SO₄ environment, 3 replicate lines from ancestry C went extinct, one during the 2nd sexual cycle, another during the 4th, and the other during the 5th, and in the Herbicides environment, 3 replicate lines from ancestry C and one replicate from ancestry B went extinct, during the 1st, 3rd, 6th, and 4th sexual cycles respectively. Attempts were made to mate them again whenever this happened but failed repeatedly in these particular cases.

Population sizes generally increased throughout the experiment, over the asexual and sexual cycles, except during the asexual cycles in Bold's where they remained stable (data not shown). The minimum population size, i.e. the lowest cell density reached by the end of a growth cycle out of all asexual cycles, was on average 4.2×10^6 , 9.8×10^5 , 2.8×10^5 , and 8.7×10^5 cells, with minimum inoculum sizes hence being 2.1×10^5 , 4.9×10^4 , 1.4×10^4 , and 4.4×10^4 cells for each line in Bold's, Herbicides, Na₂SO₄, and NaCl, respectively. The minimum population size experienced during sexual cycles was on average 2.3×10^6 , 1.1×10^6 , 5.2×10^5 , and 1.6×10^6 cells, with inoculum sizes being 1.1

x 10^5 , 5.5×10^4 , 2.6×10^4 , and 8.1×10^4 cells for lines in Bold's, Herbicides, Na_2SO_4 , and NaCl, respectively. Our protocol for selecting zygotes from sexual lines (i.e. chloroforming sexual populations to kill unmated cells) did not put the sexual lines at a disadvantage compared to the asexual lines in terms of population sizes, as there was no statistical differences between asexuals and sexuals in the minimum population size after the sexual cycle (analysis of variance: $F_{1,129} = 0.233$, $P = 0.63$).

Ancestral fitness assays

We chose maximum growth rate as our measure of fitness. Whilst in principle there are other aspects of growth dynamics that may be components of fitness, previous studies show that in experiments such as these maximum growth rate consistently shows greater selection gradients and evolutionary responses than other life-history traits such as time in lag phase and death rates at stationary phase (Dykhuizen 1990; Vasi et al. 1994; Lenski et al. 1998). Moreover, whilst maximum growth rate should always be positively selected in populations that spend reasonable amounts of time in growth phase, other aspects of fitness may vary in their direction of selection depending on the exact growth dynamics, which vary in different environments. We have nonetheless also estimated fitness using maximum optical density as a measure of yield and obtained the same qualitative results (Supplementary Figure 1 and 2).

We estimated the fitness of the ancestral spores used to assemble each selection line by measuring maximum growth rates in each of the four selection environments. The ancestors had been maintained in dim light on Bold's agar throughout the experiment,

conditions which limit growth and selection (Harris 2009). A single colony from each ancestor was grown in Bold's media for two cycles to minimise physiological differences, and then transferred in triplicate to each of the four environments. All cultures were grown for two cycles in the assay environments. Growth was monitored during the second growth cycle in the assay environments by measuring optical density at 750 nm every 8 ± 1 hours. We chose to measure during the second cycle to allow the three replicates one cycle of independent growth and avoid the measurement of initial physiological responses to the new environment.

To estimate the maximum growth rate from measures of optical density over time, we fitted a nonlinear model using nonlinear least squares in the 'nlstools' R package (Baty et al. 2015). We first fitted a baranyi model (Baranyi and Roberts 1994; Baranyi et al. 1995). This type of model fitted 90%, 94%, 67%, and 84% of spores assayed in Bold's, Herbicides, Na₂SO₄, and NaCl respectively. The fit was too poor on the other spores for the model to converge. These spores were therefore fitted using either a baranyi model without N_{max}, a baranyi model without lag, or a linear model, as appropriate. Model fits were visually inspected to ensure the proper model had been applied (for examples of a fits from each type of model see Supplementary Figure 3).

Evolved fitness assays

The evolved lines from each selection environment were assayed in their respective selection environment in separate experiments because of space constraints. For similar reasons, it was impossible for us to assay all 36 ancestral spores and all 36 evolved lines

all at once and so we assayed the fittest ancestral spore in terms of maximum growth rate, along with the evolved lines.

We assayed four random spores per evolved line. 24 spores (6 lines x 4 spores) were picked from the fittest ancestor to match the number of evolved spores assayed per ancestry x reproduction mode. All colonies were grown in Bold's liquid media for one growth cycle to minimise physiological differences, and then transferred to the environment in which the evolved lines were selected. Growth was monitored during the second cycle in the assay environment and growth parameters estimated as described above.

Statistical analyses

All analyses were performed in R version 3.2.1. To determine if the ancestral spores used to assemble the sexual lines differ from the ancestral spores used to assemble the asexual lines we fitted a mixed effect model using the lmer function in the R package 'lme4' (Bates et al. 2015). The mode of reproduction (asexual or sexual) was set as a fixed factor, while environment, ancestry, and spore within ancestry were set as random factors. P values were obtained using the R package 'lmerTest' (Kuznetsova et al. 2014) with type III sum of squares in an analysis of variance and Satterthwaite approximation for degrees of freedom by using the normal approximation.

The effect of recombination on selection was determined individually for each selection environment by fitting mixed effect models using the lmer function, with mode of

reproduction (asexual or sexual) and selection (ancestral or evolved) as fixed factors, and ancestry, line within ancestry, and spore within line within ancestry as random factors.

We allowed for random intercepts.

To estimate the constraints from ancestry, the importance of chance, and the change in diversity within lines, we calculated the difference between evolved variances and ancestral variances. Thus a positive change in variance indicates that there is more variation after evolution than at the start (i.e. divergence over time), whereas a negative change in variance indicates that there is less variance after evolution than at the start (i.e. convergence over time). The evolved variances were extracted from a model with ancestry, line within ancestry, and spore within line within ancestry as random factors. Separate models were fitted for each combination of environment and mode of reproduction. The ancestral variances were extracted from a model with ancestry and spore within ancestry as random factors. The among-line ancestral variance was set at zero. Note here that the evolved data and the ancestral data come from different fitness assays. Temporal heterogeneity in environmental conditions between assays can lead to differences in growth. It is unlikely that temporal heterogeneity would interact with the mode of reproduction treatment, and so the variance estimates for the asexuals and the sexuals should be affected to the same extent. The actual value of the change in variance is likely to be inexact, and values near zero need to be interpreted with reserve.

This approach of using the change in variance differs from our previous approach (Lachapelle et al. 2015) where we calculated the relative contribution of selection,

chance, and ancestry by dividing the evolved variance by the total evolved variance. It is only appropriate to use proportions to compare treatment levels for their effects on selection, chance, and ancestry, when the initial variance is the same across all treatment levels. For example, if lines are isogenic at the start and the same genotype is used across all treatments, then there is no need to correct for initial variance. However, in cases such as in the experiment reported here where lines are diverse at the start, and sexual and asexual lines cannot be assembled using the same genotypes (because of mating type constraints), it is not appropriate to compare evolved variances without correcting for initial variance. Differing amounts of variance can affect the potential for convergence and divergence among histories, among line, within lines. This is why we report the change in variance instead of the proportion of the total variance explained by either chance or ancestry.

To determine the statistical significance of the differences in the change in variance between asexual and sexual populations we did a randomisation test. We randomly allocated each evolved spore to a line, ancestry, and mode of reproduction (keeping spores within their environment of selection), each ancestral spore to an ancestry and mode of reproduction, and then performed the analysis described above to calculate the change in variance. The number of times the random absolute change in variance was as large or larger than the absolute observed change in variance over the total number of randomisations (10,000) is our significance statistic.

Results

We picked four different environments in which to study the consequences of sex on the repeatability of evolution. The Na_2SO_4 environment is the most severe with slowest ancestral maximum growth rates, followed by NaCl, Herbicides, and Bold's (Figure 2). To estimate the repeatability of evolution, we measured the strength of both deterministic factors such as selection, and stochastic factors such as chance and ancestry. Note that we use the terms divergence and convergence when referring to an increase and decrease in variance over time, and the term diversity when referring to the amount of variance at a given time point. Below we present the results for each environment in turn.

Repeatability of evolution in Na_2SO_4

We first determine what effect sex has on the efficiency of selection. The effect of selection is estimated by comparing the fitness of evolved spores to that of the ancestral spores, such that the greater the difference, the greater the contribution of selection to evolutionary change. The evolved lines have higher maximum growth rates than their fittest ancestral spore in Na_2SO_4 ($t_{63} = 4.79$, $P = 1.05 \times 10^{-5}$) indicating that selection has been effective in increasing fitness; and the sexual lines have increased their maximum growth rates to a greater extent than their corresponding asexual lines after selection (Figure 3; Table 1; $t_{63} = 4.22$, $P = 7.91 \times 10^{-5}$), indicating that sex increased the effect of selection.

Second, we determine what effect sex has on the constraints of ancestry. The effect of ancestry is estimated by comparing the variance among ancestries before and after

evolution, such that the greater the increase in variance among ancestries, the greater the constraints from ancestry. Ancestries diverged during evolution in Na_2SO_4 , indicating that ancestry constrained evolution; but the sexual lines diverged less than their asexual counterparts (Figure 4; $P = 0.0054$), indicating that sex reduced the constraints of ancestry.

Third, we determine what effect sex has on the importance of chance. The importance of chance is estimated by comparing the variance among replicate lines before and after evolution, such that the greater the increase in variance, the greater the importance of chance. Replicate lines diverged during evolution in Na_2SO_4 , indicating that chance had an important contribution to evolutionary change, and the sexual lines diverged more than their asexual counterparts (Figure 4; $P < 0.0001$), indicating that sex increases the importance of chance during evolution.

Finally, as our experimental lines were initially genetically diverse, we sought to determine if sex had any effect on the maintenance of diversity within lines over long evolutionary timescales. If sex helps to generate diversity within lines during evolution compared to asexual reproduction, then we should see an increase in variance among spores within a line, and if sex leads to a reduction in diversity during evolution, then we should see a decrease in variance among spores. Note that our design for the fitness assays is such that we can separate out variance among spores from variance from measurement error (see Methods). Variance among spores increased during evolution in

Na₂SO₄, and increased to a greater extent in sexual lines than in their asexual counterparts (Figure 4; $P = 0.0028$).

Hence, after 300 generations of evolution in 7 gL⁻¹ Na₂SO₄ sex appears to increase the importance of selection and chance, reduce the constraints of ancestry, and lead to greater increases in diversity within lines (Table 2). We now look at whether the effects of sex on (1) selection, (2) ancestry, (3) chance, and (4) diversity within lines observed after evolution in Na₂SO₄ are the same after evolution in NaCl, Herbicides, and Bold's in turn.

Repeatability of evolution in NaCl

The evolved lines have higher maximum growth rates than their fittest ancestral spore ($t_{66} = 6.28$, $P = 3.04 \times 10^{-8}$), and the sexual lines have higher maximum growth rates than their corresponding asexual lines after selection (Figure 3; Table 1; $t_{66} = 2.62$, $P = 0.0108$). Ancestries converged during evolution in NaCl, and sexual lines converged to the same degree as the asexual lines ($P = 0.26$). In terms of chance, replicate lines diverged during evolution in NaCl, but sex again had no measurable effect (Figure 4; $P = 0.26$). Finally, variance among spores was lower after evolution in NaCl, but decreased to the same extent in sexual and asexual lines (NaCl: $P = 0.21$). Hence, after 300 generations of evolution in 5 gL⁻¹ NaCl sex appears to increase the importance of selection, but does not alter the constraints of ancestry, the importance of chance, or the amount of diversity within lines (Table 2).

Repeatability of evolution in the herbicide mixture

The maximum growth rate of lines evolved in the herbicide mixture is no different from that of their fittest ancestral spore ($t_{62} = 0.820$, $P = 0.416$), and there is no effect of sex on selection (Figure 3; Table 1; $t_{62} = -0.386$, $P = 0.701$). Upon visual inspection, it appears that the lack of response to selection might be due to contrasting responses in each ancestry. Indeed, by analysing each ancestry on their own, we find that while for ancestry A and C sexual spores have lower fitness than asexual spores after evolution (ancestry A: $t_{20} = -2.86$; $P = 0.0098$; ancestry C: $t_{17} = -3.14$; $P = 0.0059$); for ancestry B sexual spores have higher fitness than asexual spores after evolution ($t_{19} = 3.52$; $P = 0.0023$). This effect of ancestry on the response to selection was also detected in the analyses below.

Ancestries diverged during evolution in herbicides, and sexual lines diverged more than their asexual counterparts (Figure 4; $P < 0.0001$). In terms of chance, replicate lines diverged during evolution, and sexual lines diverged less than their asexual counterparts ($P < 0.0001$). Finally, variance among spores was lower after evolution in the herbicide mixture, but decreased to the same extent in sexual and asexual lines (Herbicides: $P = 0.075$). Hence, after 300 generations of evolution in the herbicide mixture, sex appears to have no effect on the importance of selection and the amount of diversity within lines, increases the constraints of ancestry, and decreases the importance of chance (Table 2).

Repeatability of evolution in Bold's

The lines evolved in Bold's have significantly lower maximum growth rates than their fittest ancestral spore ($t_{66} = -5.38$, $P = 1.05 \times 10^{-6}$), but there is no effect of sex on selection (Figure 3; Table 1; $t_{66} = 1.61$, $P = 0.112$). The amount of variance among

ancestries remained the same during evolution in Bold's, and sex had no effect (Figure 4; $P = 0.26$). In terms of chance, replicate lines diverged during evolution, and sexual lines diverged more than their asexual counterparts ($P = 0.0084$). Finally, variance among spores was lower after evolution in Bold's, and decreased to a greater extent in sexual lines than in their asexual counterparts ($P = 0.036$). Hence, after 300 generations of evolution in Bold's sex appears to have no effect on the importance of selection or the constraints of ancestry, but increases the importance of chance, and lead to greater reductions in diversity within lines (Table 2).

Discussion

We propagated sexual and asexual lines in four different novel environments for 300 generations. By measuring the change in fitness, and the change in variance among ancestries, among replicate lines, and among spores, we were able to determine the consequences of sex on the roles of selection, ancestral constraints, and chance during evolution. We predicted that sex would increase the importance of selection and reduce the importance of chance, and so increase the repeatability of evolution, and that sex would reduce divergence among spores and so decrease diversity within populations over time.

In accord with many other experimental studies (Zeyl and Bell 1997; Colegrave 2002; Kaltz and Bell 2002; Goddard et al. 2005; Morran et al. 2009; Becks and Agrawal 2010; Lachapelle and Bell 2012; Bell 2012a), in all of our environments where maximum

growth rates increased after evolution (i.e. Na₂SO₄ and NaCl), and hence where we were able to detect an effect of selection, sex led to greater rates of adaptation. In the Bold's and Herbicides environments where maximum growth rates did not increase over evolution, there was no effect of sex.

Second, we predicted that sex would make chance associations between new mutations and genetic backgrounds less important and thus reduce the importance of chance. This second prediction is clearly not supported in all environments: sex decreased the importance of chance in the herbicide mixture, but increased the importance of chance in Na₂SO₄ and Bold's, and had no effect in NaCl. The lack of generality in the effect of sex on evolution is consistent with findings from another study of the effect of sex on the evolution of herbicide resistance in *C. reinhardtii* (Lagator et al. 2014), and in general with the variable outcomes from different studies of the repeatability of evolution in sexual species (Teotonio and Rose 2000; Teotonio et al. 2002; Kawecki and Mery 2003; Joshi et al. 2003b; Griffiths et al. 2005; Simões et al. 2008; Fragata et al. 2014).

Third, we predicted that the greater efficiency of selection in sexual populations would lead to less divergence among spores in sexual populations than in asexual populations. We indeed observed a greater reduction of diversity within sexual lines than within asexual lines in three of the four environments (Bold's, Herbicides, and NaCl). This could be because sex reduces the effects of clonal interference (Gerrish and Lenski 1998) and hence lowers the number of variants competing at any one time point. A reduction in diversity within populations could also be favoured by the type of selection regime used

in this experiment, i.e. sexual cycles interspersed by tens of generations of vegetative growth. One episode of recombination would contribute in generating variation and separating beneficial mutations from inferior backgrounds, and subsequent asexual generations would give time for selection to lead to the increase in frequency (and perhaps fixation) of the best clone (McDonald et al. 2016).

Convergence and divergence among spores over evolutionary times is rarely investigated, perhaps because most evolution experiments start with a single clone. By starting our experiment with diverse populations we were able to determine whether diversity within laboratory populations is gained or lost during evolution. We found that the magnitude of the change in variance among spores was in some environments as great or greater (e.g. in Bold's and NaCl) than the change in variance among lines or ancestries, and significantly different between asexual and sexual lines. Hence sex can have important implications for diversity not only among independent populations but also within them.

The effect of sex on repeatability depends on the environment

There are situations in which theory predicts that sex might increase divergence between adapting populations (Weinreich and Chao 2005). On a rugged fitness landscape, chance events and/or ancestry might lead a population onto a fitness peak that is less than optimal. Once that peak has been reached, all single mutations will be deleterious, and only the combination of some of these single mutations will be beneficial and take the population to another potentially higher peak. When fitness valleys are shallow, single mutants will be selected out slowly and remain in the population longer. The high

frequency of single mutants will generate negative linkage disequilibrium, meaning that recombination will tend to generate multiple mutants. Hence in such cases, sex will reduce the importance of chance by favouring peak shifts and convergence on the optimal fitness peak. On the other hand, when fitness valleys are very deep, single mutants will be selected out rapidly and very few will exist at any one time in the population. The low frequency of single mutants will generate positive linkage disequilibrium, meaning that recombination will instead tend to break apart beneficial combinations. Hence, by hindering peak shifts, sex will increase the importance of chance and history and decrease the repeatability of evolution.

The theory available therefore predicts either an increase or a decrease in variance among lines during evolution, depending on the value of a number of parameters. For example, differences in linkage disequilibrium can arise not only because of differences in the genetic basis of adaptation, but also because of differences in population size and in initial distance to fitness peaks (Otto et al. 1994; Kondrashov and Kondrashov 2001; Hadany and Beker 2003; de Visser et al. 2009). While our study did find that sex could either increase or decrease variance in fitness among populations, without precise information on the relevant parameter values for our environments (e.g. number of genes involved in fitness, ruggedness of the fitness landscape, distance to optimal fitness, etc.), we are unable to determine if the outcome in any one environment supports that made by theory. This study, which to the best of our knowledge is the first empirical attempt to test these predictions in multiple environments, indicates that different environments,

with their different parameter values can lead to vastly different outcomes, although more work is clearly needed to determine precisely which parameters are important.

The different effects of sex on evolution in different environments could be in part due to the genetic basis of adaptation as it very likely to differ among environments. Growth rates were ancestrally lowest in the Na₂SO₄ environment, followed by NaCl, Herbicides, and Bold's. Therefore, assuming that the optimal fitness in each environment is the same as fitness in the basal medium (i.e. Bold's) without the added stressor, lines would have been furthest away from the fitness peak in Na₂SO₄ and NaCl, and closest in Herbicides at the start of the experiment. This most likely explains the greater increase in fitness in these two former environments, as more beneficial mutations would have been available. Na₂SO₄ and NaCl are also likely to have the most complex genetic basis of adaptation as both impose osmotic and oxidative stresses that have been shown to require changes in many genes in *C. reinhardtii* (Perrineau et al. 2014). It is therefore unsurprising that sexual populations were at an advantage over asexual populations in these environments as recombination would be helpful in combining the many mutations together instead of waiting for each mutation to fix one after the other. On the other hand, the Herbicides environment contained two herbicides with only two primary targets for selection, photosystem II and very-long-chain fatty acid synthesis, reducing the potential for advantages to sex.

Aside from differences in the genetic basis of adaptation, differences among sexual and asexual populations in their levels of convergence or divergence could have arisen from

temporal effects. For example, divergence of adapting populations can be temporary when different populations follow different paths up the same fitness peak. It is therefore possible that given a few 1000s generations more, variance among populations that have diverged during the first 300 generations would be reduced to zero. While theory suggests that, in general, sexual populations will climb a peak faster than asexual populations (Weismann 1889; Fisher 1930; Muller 1932; Hill and Robertson 1966; Felsenstein 1974; Peck 1994; although see Kondrashov and Kondrashov 2001; Watson and Wakeley 2005), it is unclear what effect recombination will have on the diversity of paths followed by different populations on the same peak.

Finally, another factor that could have affected the amount of divergence among sexual populations is the frequency of sexual events. In our experimental populations, sexual events occurred about every 50 asexual generations. While such a rate of sexual events is representative of some organisms, others reproduce sexually at much more frequent intervals and this could have an effect on the importance of selection, chance, and ancestral constraints during evolution. A larger interval between sexual cycles leaves time for selection to lead to the increase in frequency of new beneficial combinations. We would expect in theory that this would increase the repeatability of evolution, as it prevents 'escape' genotypes (i.e. genotypes that fall on another peak than the one currently occupied by the population) from being constantly being broken down. A proper investigation of this effect is needed.

The evolution of slower growth rates in Bold's

582 Evolution in the Bold's environment led to lower growth rates than that of the fastest
583 growing ancestral spore. Bold's medium is a benign environment where growth rates are
584 high, and beneficial mutations are likely to be rare. The lower growth rates could be
585 attributable to a lack of relevant variation, inefficient sorting of the standing genetic
586 variation, a failure to remove deleterious mutations, or a trait other than maximum growth
587 rate being under selection. The response of a population to selection should be
588 proportional to the variance in fitness (Fisher 1930). Variance in fitness is initially high in
589 both the asexual and sexual lines in Bold's. As a rough estimate, for a selective advantage
590 of 0.1 (based on the variance present initially in the lines), and an initial frequency of 1/8,
591 we expect the fittest spore to rise to 99% frequency within 45 generations. Diversity of
592 growth rates was almost completely lost within both the asexual and sexual lines, which
593 is further evidence that sorting did occur. It is therefore unlikely that lack of variation or
594 inefficient sorting in the asexual and sexual lines is responsible for their lower mean
595 fitness. It is also unlikely that deleterious mutations fixed (either singly or through
596 hitchhiking) given the short evolutionary timescale (300 generations) and the relatively
597 large deleterious effect size that would be needed to produce such drop in growth rate.
598 Maximum growth rate has generally been found to be the most important component of
599 selection in microbial experimental evolution studies like the one described here where
600 populations are maintained by serial dilutions (Dykhuizen 1990; Vasi et al. 1994; Lenski
601 et al. 1998). Even when we looked at a different estimate of fitness, yield, we found a
602 decrease in yield after evolution (Supplementary Figures 1 and 2), indicating that this
603 result is not an artefact of our choice of estimate for fitness. Ultimately, we cannot
604 exclude the possibility that slower growth rates arose both in asexual and sexual lines as

an indirect result of selection on another trait with antagonistic effects on growth rates, or that selection in Bold's selects for slower growth rates instead of faster growth rates as a means to maintain cell health in favourable environments (Schaum and Collins 2014).

Conclusion

We found that sexual populations converged or diverged to a significantly different degree than asexual populations during evolution, reflecting differences in the importance of chance and ancestral constraints. The effects of sex on evolution are highly dependent on the genetic background and the environment, and we therefore cannot assume that results from experiments with a single genotype or environment will generalise to other environments. More rigorous tests are needed to determine the exact mechanisms by which population and environmental attributes mediate the effect of recombination. While the effects of sex on rates of adaptation and variance within populations are well appreciated, by focussing on changes in variance among populations, we have found that sex also has important downstream consequences on diversity among populations and on the predictability of evolution.

Acknowledgements

We thank S. Kraemer and reviewers for comments on the manuscript. JL was funded by the Natural Sciences and Engineering Research Council of Canada and a studentship

from the School of Biological Sciences, University of Edinburgh. NC is funded by the
Biotechnology and Biological Sciences Research Council UK.

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782

783 **Tables**

784 Table 1. The effect of recombination on the efficiency of selection at increasing
785 maximum growth rates in each of the four selection environments. The parameter
786 estimates for the fixed effect are shown, where ‘Selection’ has two levels (ancestral and
787 evolved) and ‘Reproduction’ has two levels (asexual and sexual).

Environment	Effect	Estimate	SE
Bold’s	Intercept	4.9	0.22

	Selection (evolved)	-1.4	0.26
	Reproduction (sexual)	-0.63	0.26
	Selection (evolved) : Reproduction		
	(sexual)	0.60	0.37
Herbicides	Intercept	2.7	0.23
	Selection (evolved)	0.16	0.20
	Reproduction (sexual)	-0.11	0.20
	Selection (evolved) : Reproduction		
	(sexual)	-0.11	0.29
Na ₂ SO ₄	Intercept	1.2	0.11
	Selection (evolved)	0.56	0.12
	Reproduction (sexual)	-0.096	0.12
	Selection (evolved) : Reproduction		
	(sexual)	0.71	0.17
NaCl	Intercept	1.6	0.24
	Selection (evolved)	0.85	0.14
	Reproduction (sexual)	0.068	0.14
	Selection (evolved) : Reproduction		
	(sexual)	0.51	0.19

Table 2. The effect of sex on the contribution of selection, the constraints of ancestry, and the importance of chance to evolution; and the effect of sex on the amount of diversity within lines. An upward pointing arrow indicates that the component is significantly

greater in sexual populations than in asexual populations (e.g. the constraints of ancestry are greater in sexual populations than in asexual ones in the Na₂SO₄ environment), a downward pointing arrow indicates that the component is significantly lower in sexual populations than in asexual populations (e.g. there is a greater reduction of diversity in sexual populations than in asexual ones in the Bold's environment), an equal sign indicates that there are no significant differences between sexual and asexual populations for that component.

Environment	Selection	Ancestry	Chance	Diversity
Na ₂ SO ₄	↑	↓	↑	↑
NaCl	↑	=	=	=
Herbicides	=	↑	↓	=
Bold's	=	=	↑	↓

Figure legends

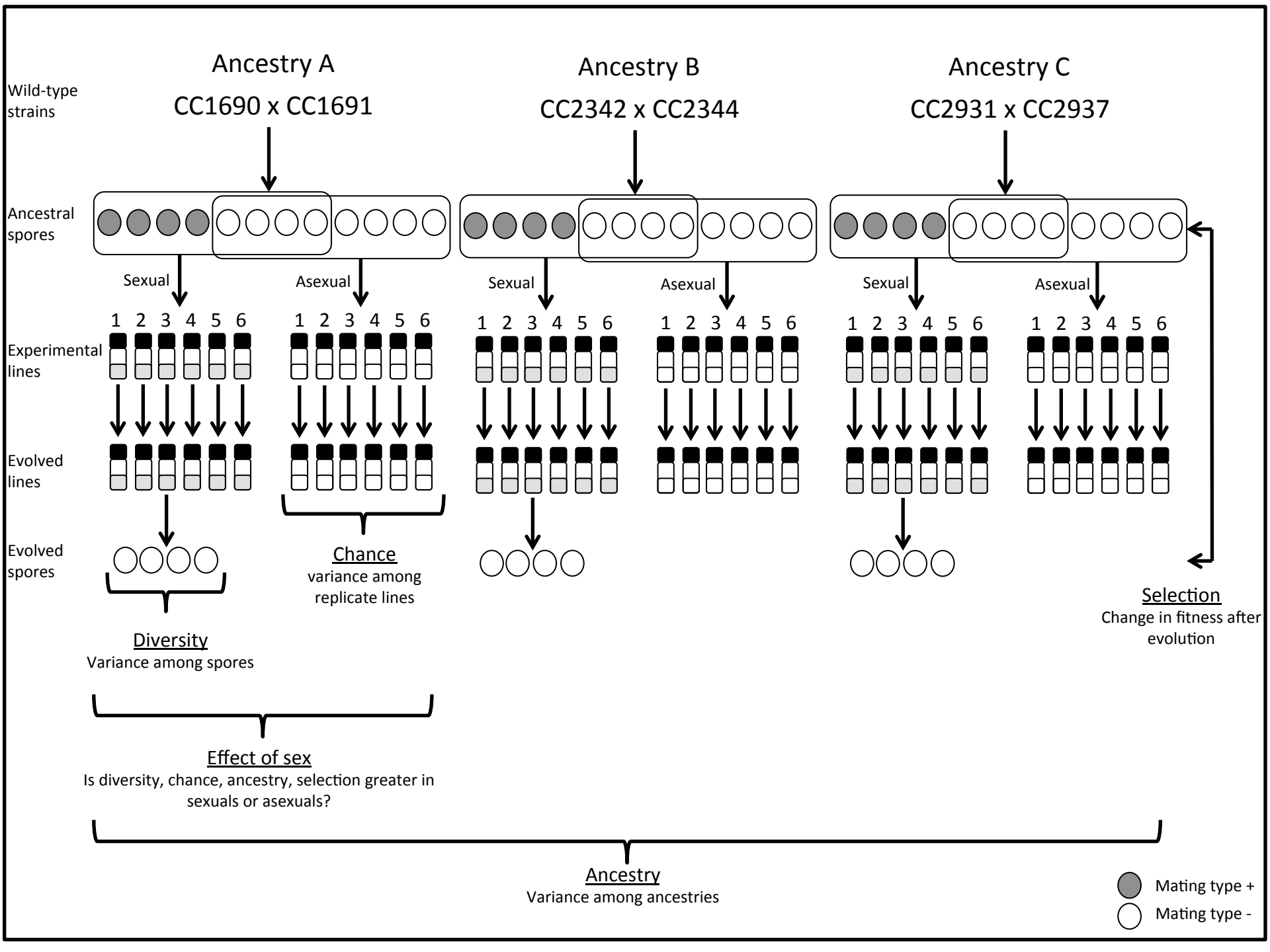
Figure 1. Schematic of the experimental design showing the original crosses that yielded the ancestral spores for each of the different ancestries, the replicate experimental lines, and the sexual treatment. The setup was replicated four times in four different environments: Bold's minimal medium, Herbicides, Na₂SO₄, and NaCl. Four spores from each of the evolved lines were assayed, but only one set for each treatment is shown in this schematic.

Figure 2. Maximum growth rate of the twelve ancestral spores from each ancestry, in each of the four selection environments. Each point represents the average of the three

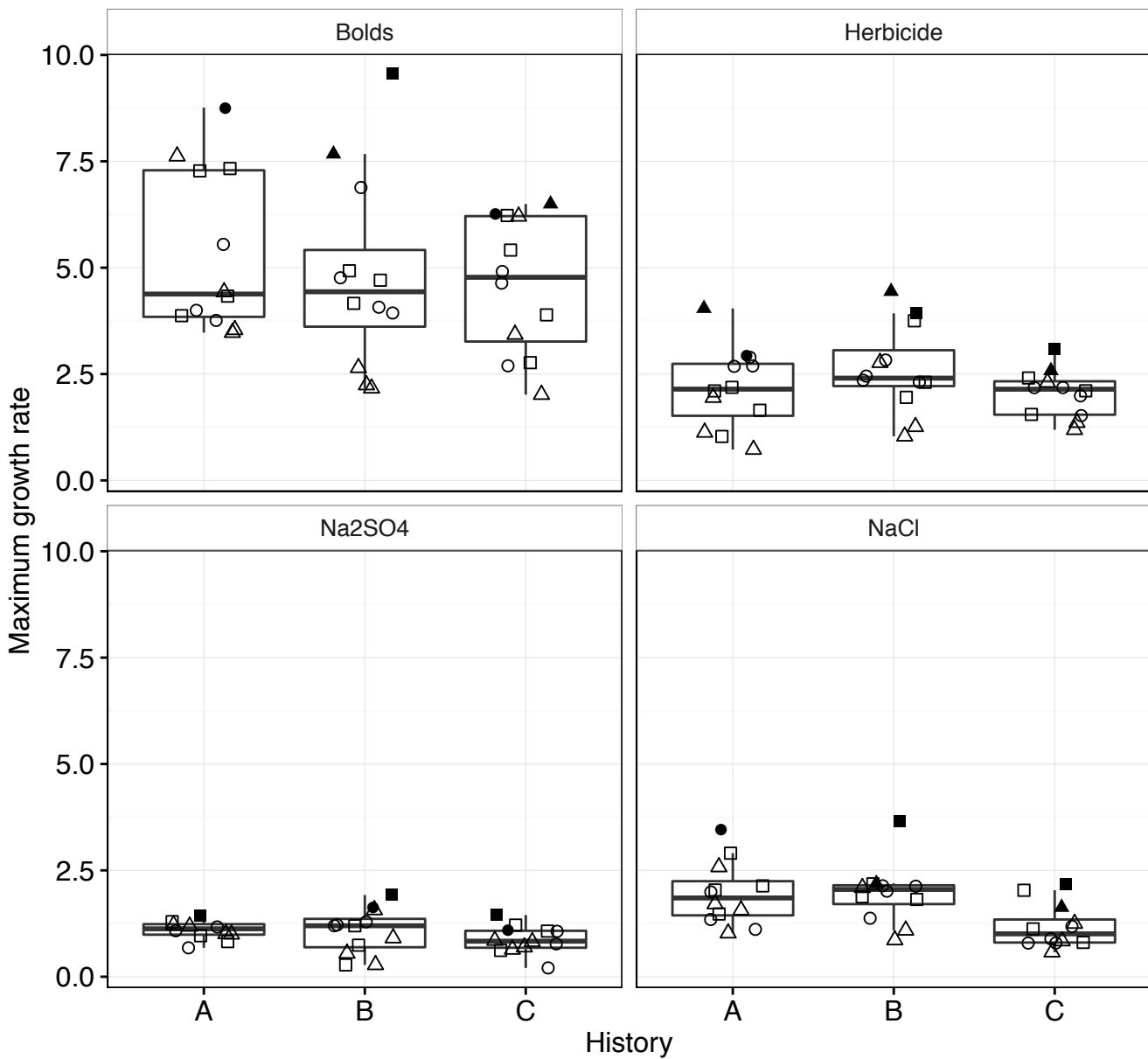
assay replicates. The shape of the points indicates whether the spore was used to found the asexual lines, sexual lines, or both. Filled points indicate the fastest growing ancestral spores used in the evolved fitness assays.

Figure 3. The effect of sex on selection. This plot shows the maximum growth rate of the fastest growing ancestral spores and the evolved spores in their corresponding selection environment. The difference in maximum growth rate between evolved and ancestral indicates the effect of selection. A difference in the magnitude of this change indicates the effect of sex. Each point represents the average of the three assay replicates. There are 4 spores for each of 36 evolved lines (except in Herbicides where there are 32 lines and in Na_2SO_4 where there are 33 lines). The shape of the points indicates from which ancestry the spore comes from.

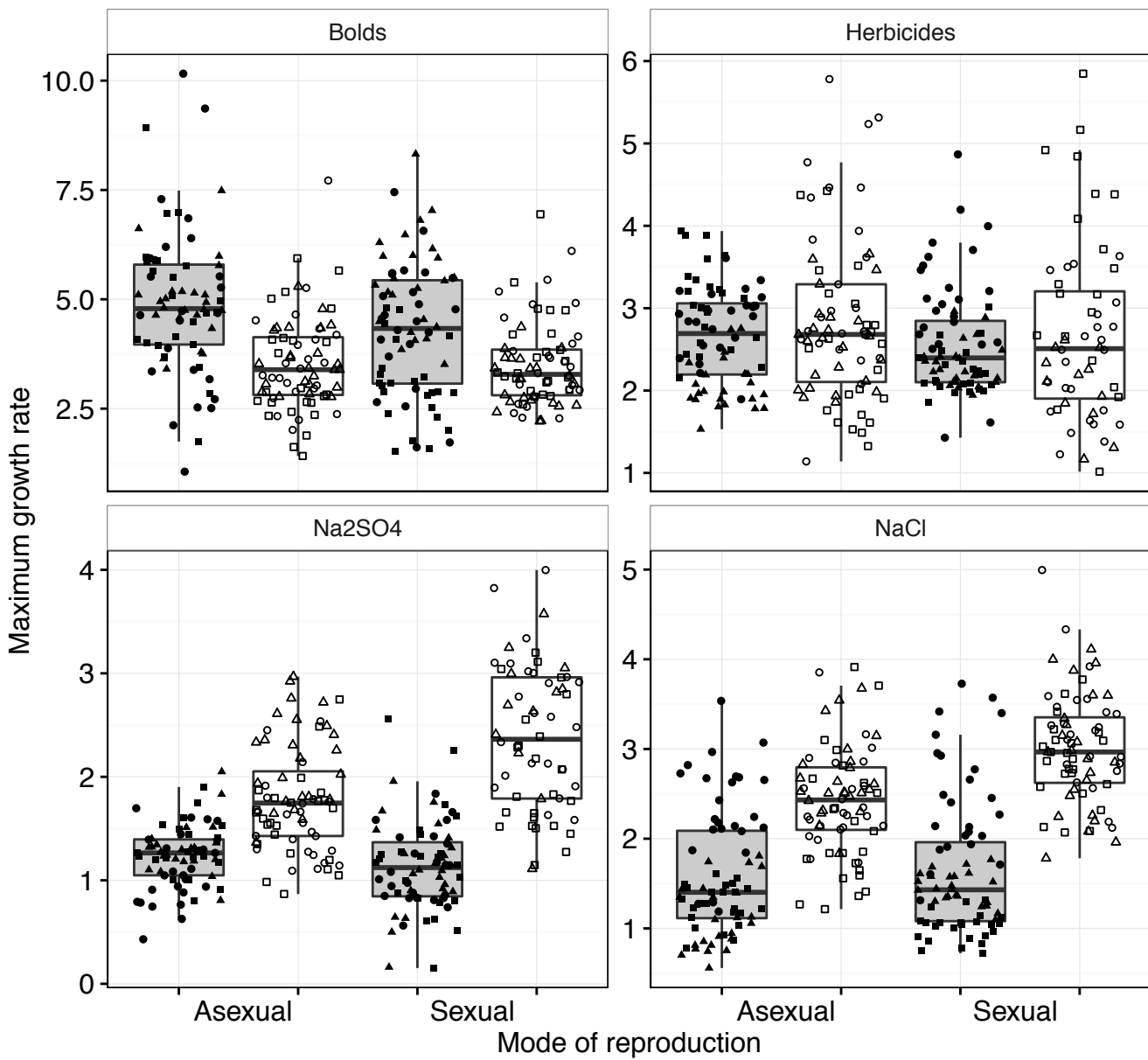
Figure 4. Change in variance in maximum growth rate after evolution in each selection environment in asexual and sexual populations. Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.



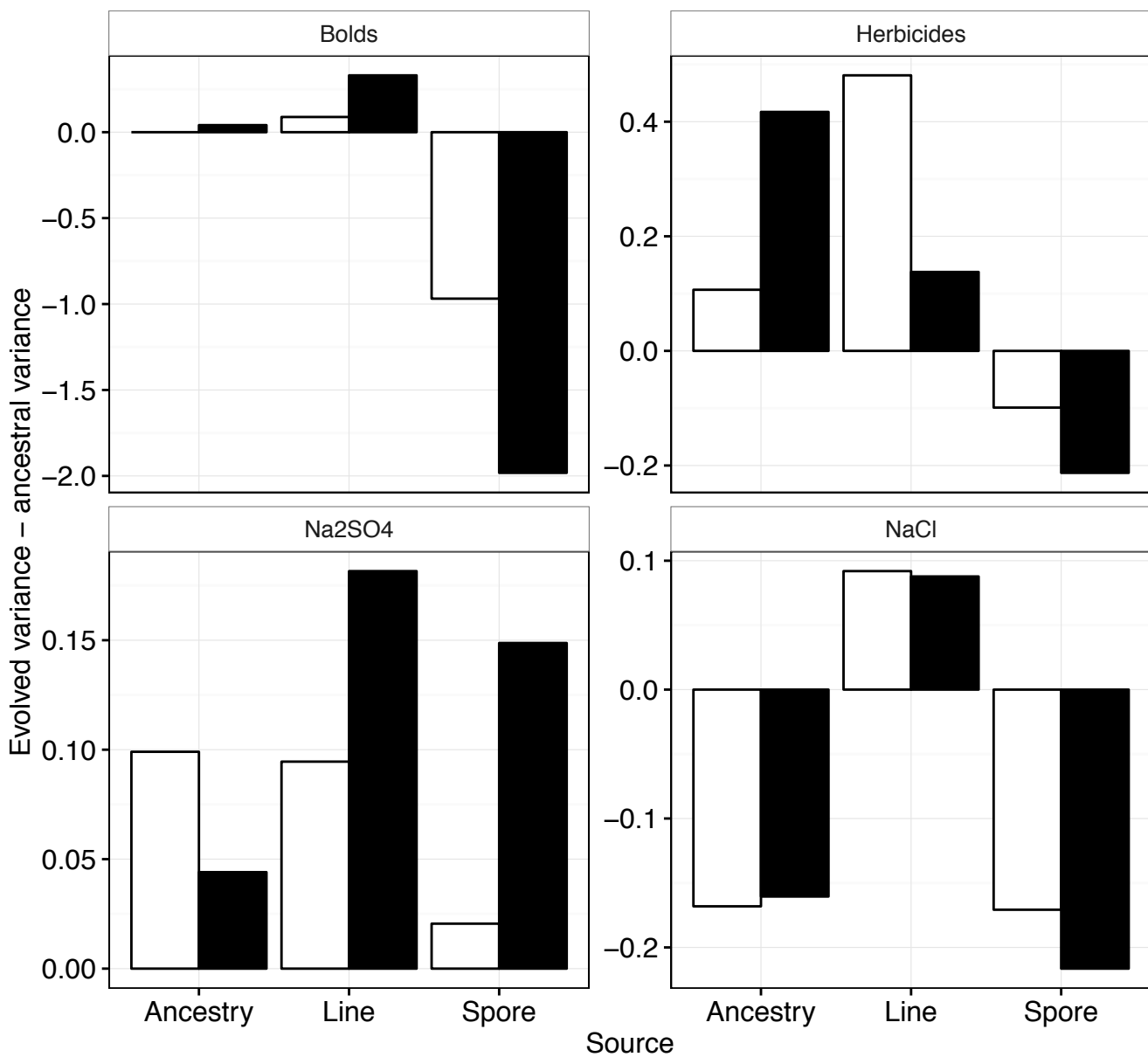
Ancestral spore for □ asexual lines ○ both asexual and sexual lines △ sexual lines



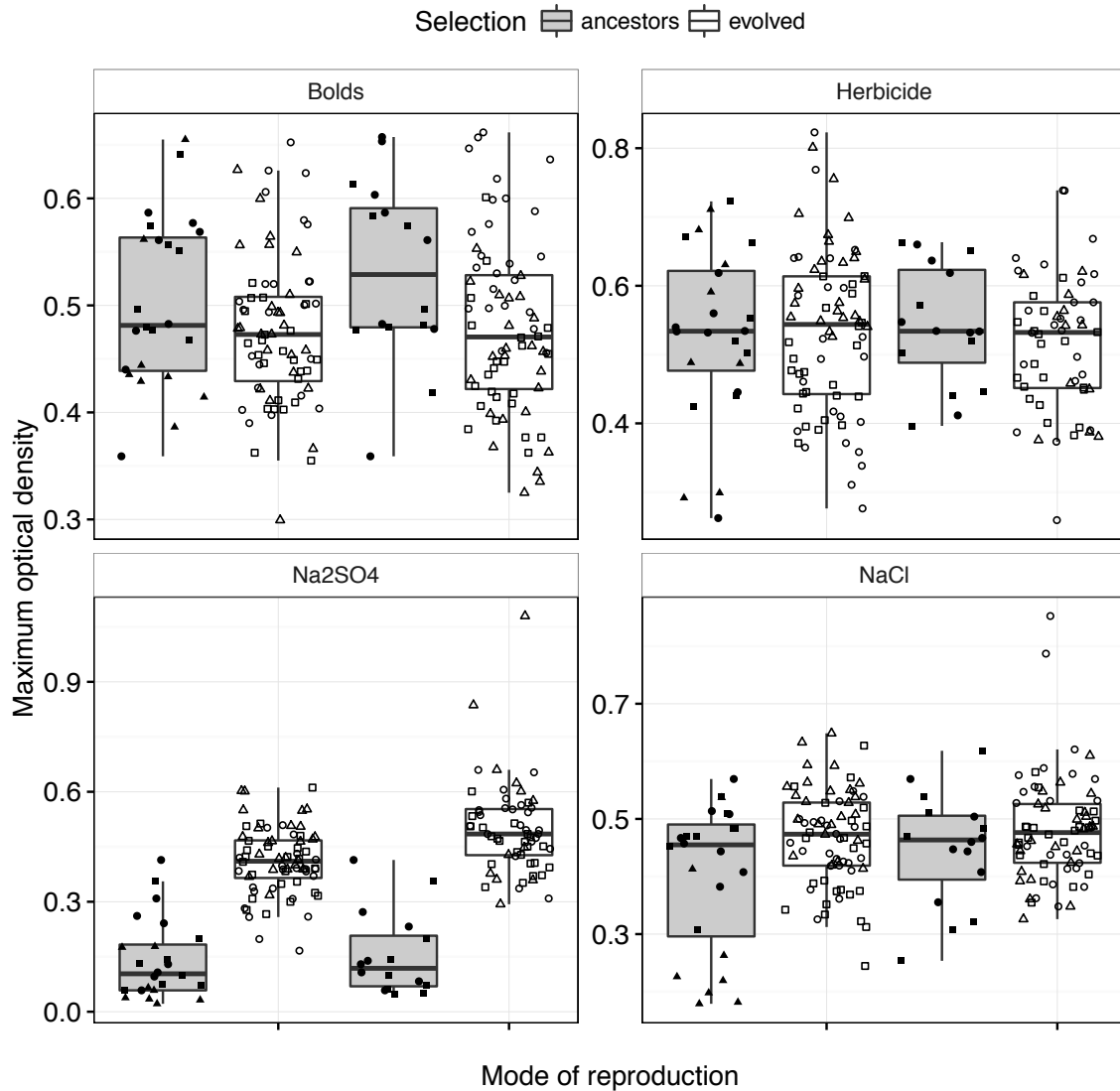
Selection  ancestor  evolved Ancestry  A  B  C



Reproduction asex sex

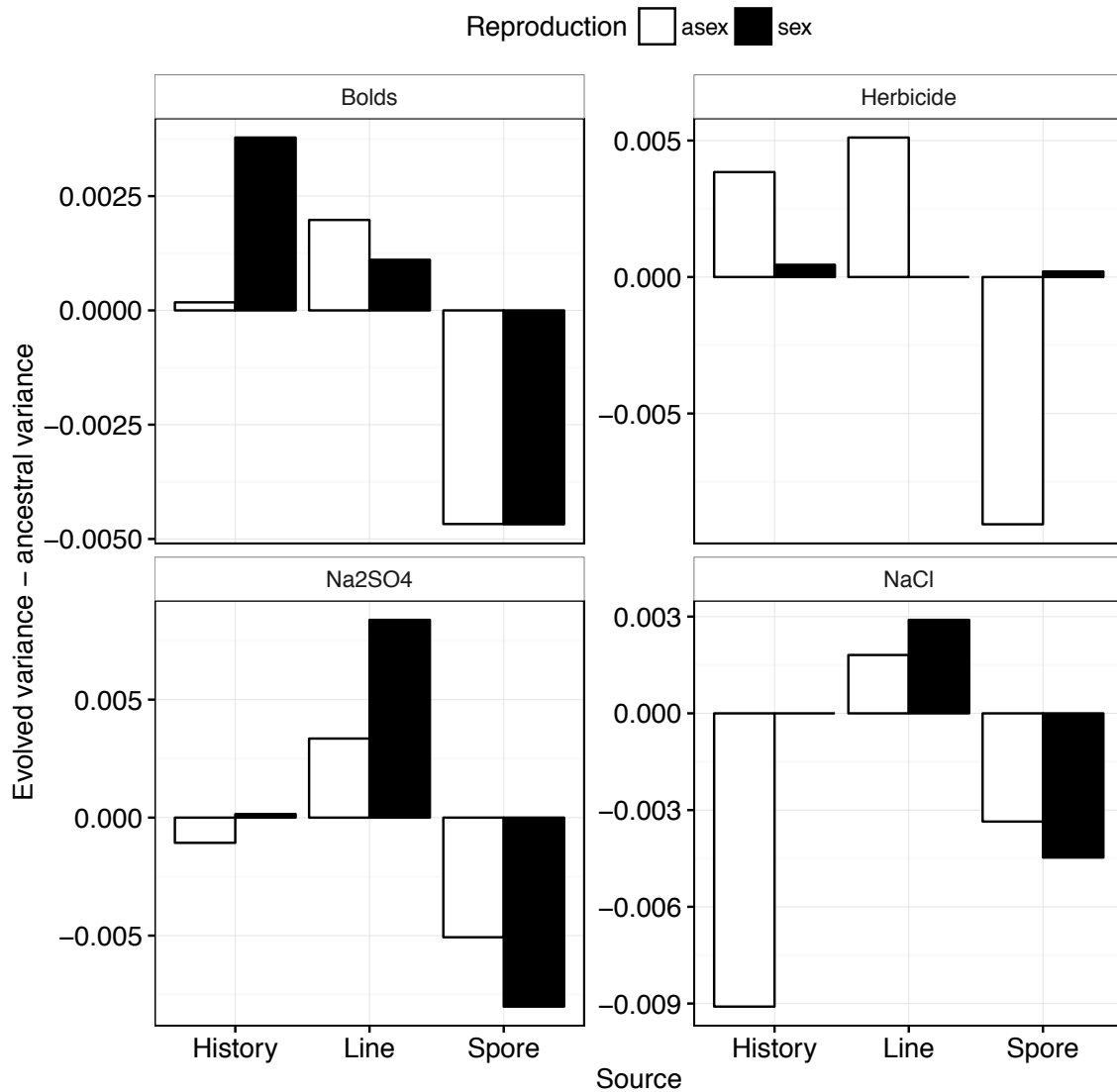


Supplementary Figures



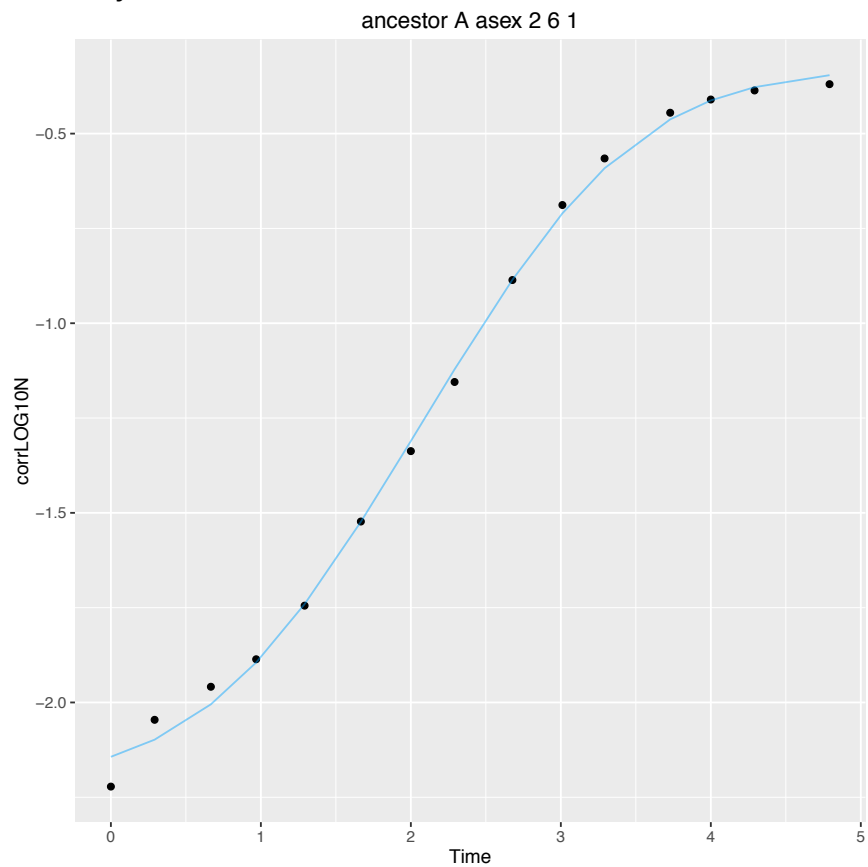
Supplementary Figure 1. The effect of sex on selection. This plot shows the yield of the ancestral spores and the evolved spores in their corresponding selection environment. The difference in yield between evolved and ancestral indicates the effect of selection. A difference in the magnitude of this change indicates the effect of sex. Each point represents the average of the three assay replicates. There are 4 spores for each of 36 evolved lines (except in Herbicides where there are 32 lines and in Na₂SO₄ where there

are 33 lines). The shape of the points indicates from which ancestry the spore comes from.

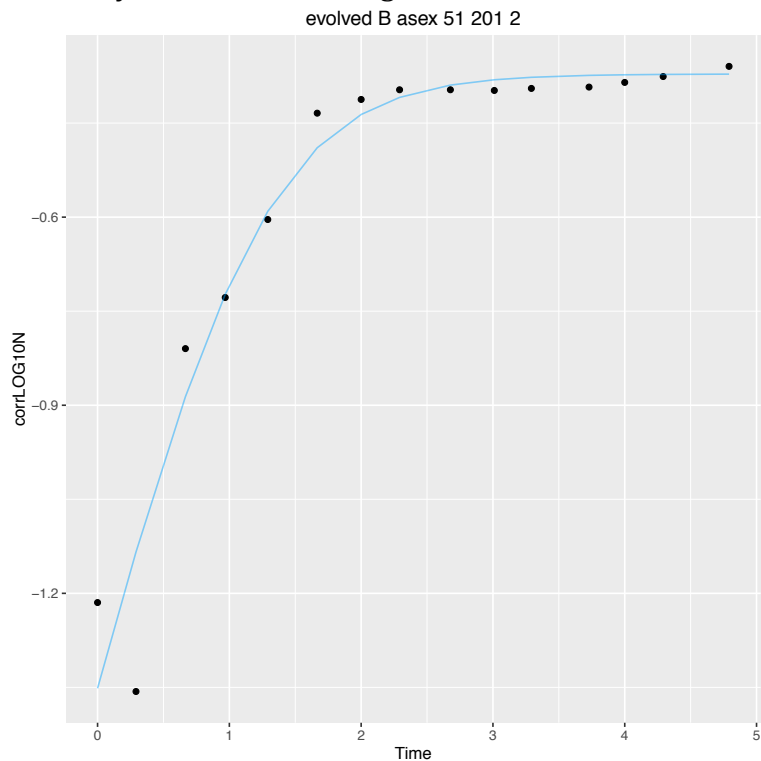


Supplementary Figure 2. Change in variance in yield after evolution in each selection environment in asexual and sexual populations. Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.

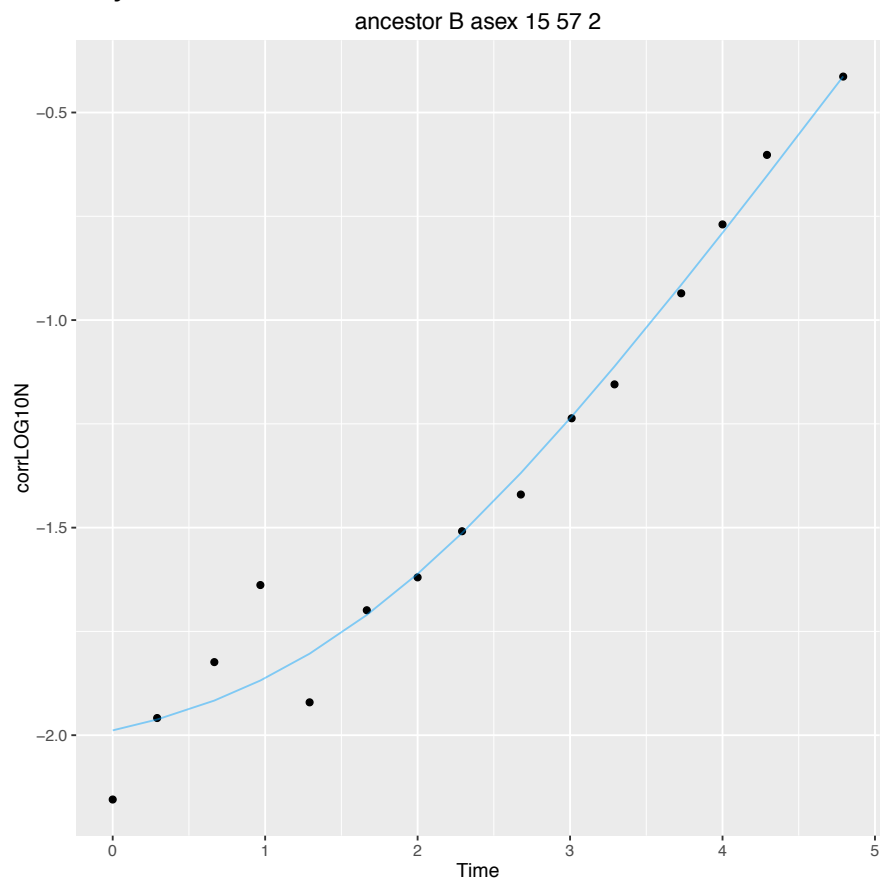
A. Baranyi model



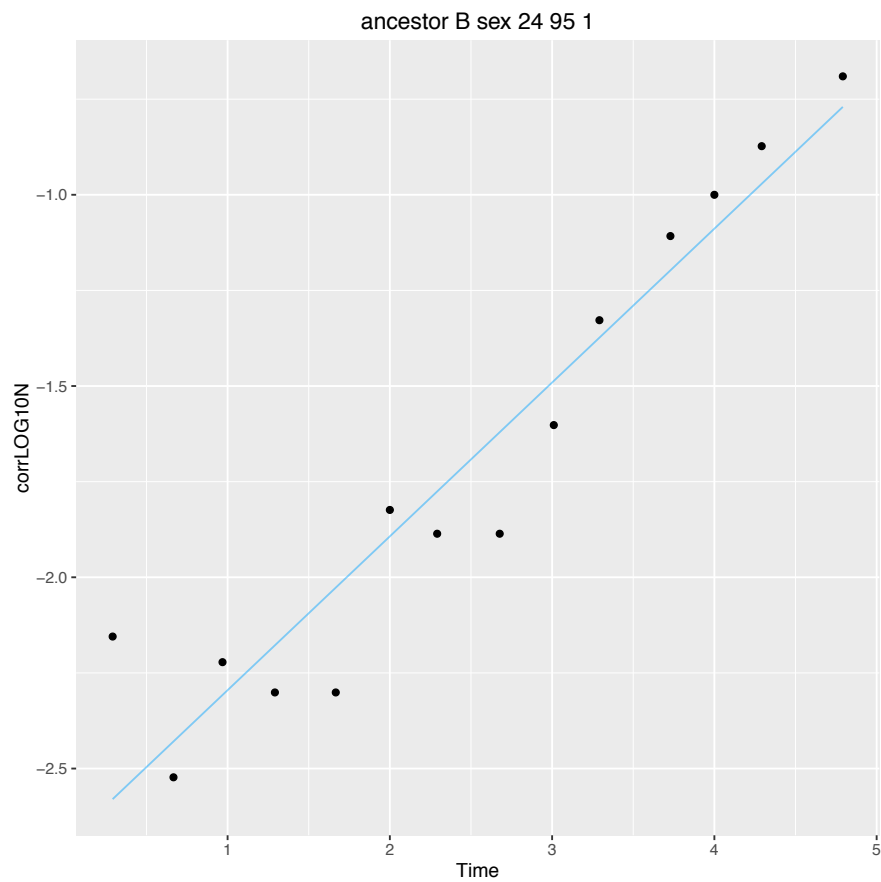
B. Baranyi model without lag



C. Baranyi model without Nmax



D. Linear model



Supplementary Figure 3. Examples of spores grown in 5 gL⁻¹ NaCl and fitted with the (A) Baranyi model, (B) Baranyi model without lag, (C) Baranyi model without Nmax, and (D) linear model. The points represent the actual data, and the blue line represents the model fit.